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The central hypothesis in this IDEA Award is that increased integrin-mediated adhesiveness and migration of breast cancer cells in response to stimulation by the growth factors heregulinβ (HRGβ) or epidermal growth factor is mediated by phosphoinositide 3-OH kinase (PI 3-K)-dependent activation and membrane recruitment of the novel Tec family tyrosine kinase Etk. During the past year, we have made progress in several aspects of the initial Statement of Work, including: 1) establishment of a correlation between Etk expression and several breast tumor cell lines with high migratory capacity; 2) demonstration of HRGβ-dependent increases in tyrosine phosphorylation of Etk in breast cancer cells; 3) demonstration of binding of the PH domain of Etk to the PI 3-K lipid product PI(3,4,5)-$P_3$; 4) demonstration that HRGβ stimulation leads to enhanced membrane localization of Etk that requires the PH domain of Etk and PI 3-K activity; and 5) production and establishment of molecular reagents and methodologies critical to completion of the Statement of Work.

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## INTRODUCTION

Breast cancer cell metastasis is critically dependent on integrin receptors, which mediate adhesion and subsequent cell migration [1-3]. Although quantitative changes in the level of expression of specific integrins have been implicated in breast cancer development, it is now clear that qualitative regulation of the functional activity of breast cancer cells also plays a critical role in regulating breast cancer cell adhesion and migration [4,5]. We have previously shown that stimulation of breast cancer cells with the growth factor heregulin-β (HRGβ) leads to a rapid increase in integrin-mediated adhesion of these cells to type IV collagen and laminin, and subsequent migration through collagen- and laminin-coated filters [4]. HRGβ also leads to potent activation of the lipid kinase phosphoinositide 3-OH kinase (PI 3-K), and inhibition of PI 3-K with chemical or genetic inhibitors blocks HRGβ-induced increases in integrin-dependent adhesion and migration of breast cancer cells. Similar responses were observed following stimulation of breast cancer cells with epidermal growth factor (EGF), although the magnitude of the responses were not as high as those observed with HRGβ. Similar effects of HRGβ on PI 3-K activity and migration of breast cancer cells have been reported by other groups [6,7]. In this IDEA Award, we are testing the hypothesis that increased integrin-mediated adhesiveness and migration of breast cancer cells in response to HRGβ or EGF is mediated by PI 3-K-dependent activation and membrane recruitment of the novel Tec family tyrosine kinase Etk. The specific objectives of the proposal include: 1) determining the role of PI 3-K in HRGβ- and EGF-induced activation of Etk in breast cancer cells; 2) determining the role of Etk in regulating HRGβ- and EGF-induced increases in β1 integrin-dependent adhesion and migration of breast cancer cells; and 3) identifying a function for Etk in regulating HRGβ- and EGF-induced actin polymerization in breast cancer cells.

## BODY

### EXPERIMENTAL METHODS

*Cell lines.* The MDA-MB-435s cells were maintained in RPMI medium (Gibco) supplemented with 10% fetal calf serum (FCS, Atlanta Biologicals). MCF-7 cells were maintained in RPMI medium supplemented with 10% FCS, 1 mM non-essential amino acids, sodium pyruvate and 10 μg/ml insulin. All cell lines were obtained from ATCC and all cell culture media were supplemented with 2 mM L-glutamine, and 50 U/ml penicillin/streptomycin (Mediatech).

*DNA constructs and transfections.* Etk constructs (wt, ΔPH, PH, E42K, KQ and DN) were cloned in frame into a pEGFP (Clontech) or pIRES-GFP vector (Clontech) with a T7 epitope tag. These plasmid DNA constructs were transfected into MDA-MB-435 or MCF-7 cell lines by electroporation in 0.4 cm cuvettes (Invitrogen), at 245V, for 2 pulses at 24ms using a square wave electroporator (BTX Genetronics Inc. San Diego CA). Typically, 10-20 million cells in 600 μl –800 μl of Opti MEM (reduced serum modification of minimal essential media, GIBCO) were transfected with 100-150 μg DNA. Cells were then cultured overnight in 100 $cm^2$ tissue culture plates in RPMI containing 20% BCS, 20 mM L-glutamine and 50 IU penicillin streptomycin (20% BCS/RPMI).

*Flow cytometry.* Single-color flow cytometric analysis (FACS) was performed on cells in suspension after removal from tissue culture flasks with 1 mM EDTA or 1X trypsin. 5 X $10^5$ cells were typically analyzed in FACS buffer [Hanks buffered saline solution (HBSS),

containing 1% bovine calf serum (BCS; Hyclone Laboratories, Inc)]. After 2 washes in ice-cold FACS buffer, data was acquired on a Becton Dickinson FACScan or FACScalibur and analyzed using Cellquest software.

*Immunoprecipitation.* MDA-MB-435s cells were grown to ~75% confluence in 100 $cm^2$ tissue culture dishes. For growth factor stimulation experiments, cells were serum starved overnight before stimulation. Cell lysates were prepared in RIPA buffer (1% Triton X-100, 1% deoxycholic acid, 150 mM NaCl, 5 mM EDTA, 10 mM Tris pH 7.2 containing 1 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 2 mM sodium orthovanadate). Cell lysis was performed on ice for 20 min and the lysates were centrifuged at 2000 rpm for 5 min to remove cell debris. Immunoprecipitation was performed at 4°C overnight using polyclonal Etk antibodies, anti SH Etk (Y. Qiu) or T7 tag monoclonal antibody (Novagen). The immunoprecipitates were incubated with Protein-A sepaharose beads for an additional 1 hour at 4° C. The resultant immunocomplexes were washed three times with ice cold RIPA buffer and then boiled for 5 min. in 2x sodium dodecyl sulfate (SDS) buffer (125 mM Tris pH 6.8 containing 4% SDS, 2 mM EDTA, 20% glycerol, 10% mercaptoethanol, 0.6% bromophemol blue). The samples were centrifuged at 13,000 rpm for 2 min and the supernatant was separated on a 7.5% or 10% gel by polyacrylamide gel electrophoresis.

*Western blotting.* Cell lysates or immunoprecipitates were separated by SDS-PAGE as described above and transferred to Immobilon-P membrane (Millipore) in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, 0.0075% SDS) for 2 hours at 400 mA. Membranes were incubated in blocking buffer (5% Carnation milk in PBS) or for blots used for the detection of phosphotyrosine, 1% BSA in Tris buffer (10 mM Tris pH 7.5, 100 mM NaCl) for 1 hour at room temperature or overnight at 4°C. Blots were washed in PBS prior to addition of primary antibodies diluted in blocking buffer (4G10; UBI) for 1 hour at room temperature. Blots were rinsed 3 times in PBS, 0.1% Tween-20 for 10 minutes each before addition of secondary antibodies diluted in blocking buffer [goat anti-mouse IgG-horse radish peroxidase (GAM-IgG-HRP), Gibco] for 1 hour at room temperature. Blots were rinsed 3 times in PBS, 0.1% TWEEN-20 and bands were visualized using enhanced chemiluminescence (Pierce Chemical). For re-probing membranes, stripping buffer (62.5 mM Tris, pH 6.8, 2% SDS, 0.1 M 2-ME) was used at 55°C for 30 minutes followed by blocking membranes in 5% milk, PBS, and re-probing with appropriate antibodies.

*Nitrocellulose phospholipid binding assays.* GST-Etk fusion protein binding to phospholipids was performed as previously established in our laboratory [8]. Briefly, phosphoinositides (PI, PI(3)-P, PI(4)-P, PI(4,5)-$P_2$ and PI(3,4,5)-$P_3$) were spotted onto nitrocellulose membranes and allowed to dry at room temperature for 1 hour. The membranes were then blocked for 2 hours in 3% fatty acid free bovine serum albumin in TBST (50mM Tris pH 7.5, 150 mM NaCl, 0.5 % Tween–20]. Either GST alone or GST- PH Etk, GST-E42K, and GST-R29N Etk fusion proteins at a concentration of 1 µg /ml were incubated with the membrane overnight at 4°C. The membranes were washed three times in TBST and incubated with anti-GST monoclonal antibody (Zymed) for 2 hours at room temperature, then washed three times. The membranes were incubated with a HRP-conjugated goat anti mouse IgG (Transduction Labs) for 1 hour, washed three times in TBST and the membranes were developed by chemiluminescence western blotting reagent (Pierce).

*Cellular fractionation.* Cell fractionation was performed as previously described by our lab and others [8,9] and adapted for breast cancer cell lines. MDA-MB-435 cells were grown to

confluence in 100 $cm^2$ petri dishes and serum starved overnight. The cells were then treated for 10 min at 37 °C with 100 ng/ml HRG β or 100 ng/ml HRGβ and 50 nM wortmannin or 25 μM LY294,002. Cells were then scraped off the plates in 0.75ml hypotonic buffer (19 mM Tris pH 8.0 and 1 mM $MgCl_2$ containing 0.1 mM NaOVO4, 0.1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin and 1μg/ml leupeptin). The cells were sonicated for 3 min then NaCl was added to a final concentration of 150 mM. The cell lysates were centrifuged at 200 x g for 10 min at 4 °C and the supernatant was transferred into pre-cooled ultracentrifuge tubes containing 0.1X (vol/vol) cytosolic adjusting buffer (1% Triton X-100, 1 % SDS and 1% sodium deoxycholate). The samples were centrifuged at 100,000 x g for 45 min at 4°C and the supernatant was frozen at –70°C for later determination of cytosolic Etk. The membrane pellet was re-suspended in 0.45 ml of MES buffer (25 mM MES, pH 6.5, 150 mM NaCl) containing protease inhibitors as described above. The re-suspended membrane fraction was incubated on ice for 30 min and mixed every 10 min. Equal aliquots of the membrane fractions were boiled for 5 min. in 2 x sodium dodecyl sulfate (SDS) buffer (125 mM Tris pH 6.8 containing 4% SDS, 2 mM EDTA, 20% glycerol, 10% mercaptoethanol, 0.6% bromophemol blue). The samples were separated on a 10% SDS-polyacrylamide gel and blotted for Etk using an Etk monoclonal antibody (Transduction Labs). The membranes were developed using a chemiluminesence western blotting reagent (Pierce).

*Confocal microscopy.* MCF-7 cells were transiently transfected with GFP-Etk fusion protein constructs by electroporation and seeded onto poly-L-lysine coated coverslips in a 100 $cm^2$ petri dish. Cells were allowed to recover for 48 hours following transfection and were then serum starved overnight. Growth factor stimulation was carried out at 37° C for 10 min using 100 ng/ml HRGβ in PBS/0.1% BSA. The cells were fixed in 3.7% paraformaldehyde for 10 min at room temperature. The coverslips were mounted on slides used 15% glycerol in PBS or VECTASHIELD (Vector Labs). Confocal microscopy was performed using a Bio-Rad MRC 1024 confocal microscope.

## RESULTS AND DISCUSSION

This project is investigating the role of the Tec family tyrosine kinase Etk in mediating the pro-adhesive and pro-migratory effects of the growth factor HRGβ on breast cancer cells. We specifically hypothesize that HRGβ results in PI 3-K-dependent activation of Etk, which subsequently regulates the actin cytoskeleton, potentially via interactions with the actin regulatory protein N-WASP. Activation of Etk and other Tec family tyrosine kinases, notably Itk and Btk, is regulated by PI 3-K [10-12], which produces membrane phospholipid products that recruit these kinases to the plasma membrane [13,14] . Recent studies from our laboratory have demonstrated a function for the Tec family tyrosine kinase Itk in the regulation of β1 integrin functional activity on T cells by the antigen-specific CD3/T cell receptor complex [8]. In addition, there is growing evidence that Tec family kinases play a key role in regulating the actin cytoskeleton [15,16].

The following report details our progress in each task outlined in the approved Statement of Work. During the second year of this work, we have focused our efforts on completing Aims 1 and 2 of the approved Statement of Work. Specifically, we: 1) established tyrosine phosphorylation and activation of Etk by HRGβ as well as the effects of HRGβ-mediated activation of PI 3-K on Etk activation; 2) extended these studies to include characterization of Etk activation by HRGβ in metastatic and non-metastatic breast cancer cell lines; 3) examined the effect of HRGβ on transiently expressed Etk in the non-metastatic cell line, MCF-7; 4)

examined the cellular localization of Etk following HRGβ stimulation of the breast cancer cell lines MDA-MB-435 and MCF-7; 5) determined the lipid binding characteristics of Etk fusion proteins.

AIM 1. TO DETERMINE THE EFFECTS OF HRGβ- AND EGF- MEDIATED ACTIVATION OF PI 3-K ON 1) TYROSINE PHOSPHORYLATION AND ACTIVATION OF ETK: AND 2) MEMBRANE RELOCALIZATION OF ETK.

- Analyze the effects of HRGβ and EGF on the tyrosine phosphorylation and activation of Etk.

In the first year of this work, we demonstrated that the metastatic breast cancer cell line MDA-MB-435s expresses Etk, while the non-metastatic breast cancer cell line MCF-7 does not express Etk. During the past year, we extended these studies and examined Etk expression in several other breast tumor cell lines. Figure 1 shows that the metastatic cell lines MDA-MB-435s, and MDA- MB-231 express Etk while the non-metastatic breast cancer cell lines MCF-7, T47D and SKBR3 all lack detectable levels of Etk. This provides additional evidence of a potential relationship between expression of Etk and the migratory capacity of breast cancer cells. In addition to the approved work on MDA-MB-435 cells, we performed some additional studies with MCF-7 cells, which lack Etk but express c-ErbB2, c-ErbB3 and c-ErbB4 at levels comparable to MDA-MB-435 cells (Fig. 2). We completed the analysis of the kinetics of HRGβ-induced tyrosine phosphorylation of endogenous Etk phosphorylation in MDA-MB-435 cells. Our results show increased Etk phosphorylation over time with maximal phosphorylation observed at 10 min of HRGβ stimulation (Fig. 3). In addition, we transiently transfected MCF-7 cells with T7-tagged wild-type and dominant-negative Etk. In preliminary results, we observed that HRGβ stimulation also resulted in tyrosine phosphorylation of wild-type Etk, but not dominant-negative Etk, in MCF-7 cells (Fig. 4). Together, these results suggest that HRGβ activates Etk in breast cancer cells.

- Determine the effects of PI 3-K inhibitors and dominant negative p85 subunit of PI 3-K on HRGβ- and EGF-induced tyrosine phosphorylation and activation of Etk.

As outlined in the approved Statement of Work, we have utilized the PI 3-K inhibitors wortmannin and LY 294,002 to determine the function of PI 3-K in HRGβ-mediated tyrosine phosphorylation of Etk. Preliminary results obtained to date show that treatment of MDA-MB-435 cells with wortmannin or LY294,002 inhibited HRGβ-induced tyrosine phosphorylation of Etk (data not shown). Studies are in progress to determine the effects of expression of a dominant negative form of the p85 subunit of PI 3-K on HRGβ-mediated tyrosine phosphorylation of Etk.

- Determine the phospholipid binding properties of Etk and the role of the PH domain of Etk in binding to phospholipids.

We determined the phospholipid binding properties of Etk by spotting purified phospholipids on nitrocellulose membranes and assessing binding of GST-Etk fusion proteins, as previously performed by our laboratory with the related tyrosine kinase Itk [8]. These studies show that the PH domain of Etk interacts strongly with PI(3,4,5)-$P_3$, the primary lipid product produced by active PI 3-K in cells. Some weak binding of the PH domain of Etk to PI(4)-P was also observed, while no binding to either PI(3)-P or PI(4,5)-$P_2$ was detected (Fig. 5). Mutation of the Etk PH domain at amino acid 29 (R29N) blocked phospholipid binding of Etk in this assay,

consistent with studies demonstrating that a similar amino acid substitution in the related tyrosine kinase Btk abrogates binding of Btk to PI(3,4,5)-$P_3$ [17]. The glutamic acid residue at position 42 of the Etk PH domain may be critical for interaction with focal adhesion kinase (FAK) [18] and mutation of the corresponding residue in Btk results in constitutive membrane targeting of Btk [19,20]. In our lipid binding assay, the E42K mutation in Etk did not alter binding of the GST-Etk fusion protein to PI(3,4,5)-$P_3$. However, this mutation appeared to enhance binding of Etk to PI(3)-P.

- Analyze membrane recruitment of Etk upon HRGβ and EGF stimulation by membrane fractionation techniques and confocal microscopy.

We have begun the approved cell fractionation experiments to determine the localization of Etk following HRGβ stimulation. Over the past year, we have been working on optimizing the protocols for preparing membrane and cytosolic fractions from MDA-MB-435 cells [8,9]. In addition, we are developing the protocols necessary for assessing Etk localization to detergent-insoluble membrane microdomains in MDA-MB-435 cells. Development of these protocols has taken slightly longer than expected. However, preliminary studies have shown that while there is a basal level of Etk in membrane fractions prepared from MDA-MB-435 cells, HRGβ stimulation resulted in increased membrane-localized Etk (Fig. 6).

Membrane localization of Etk was also assessed by confocal microscopy using MCF-7 cells expressing GFP or GFP-Etk fusion proteins. This approach provides us with a unique opportunity to visualize changes in HRGβ-mediated membrane localization of Etk over real time. In initial experiments, MCF-7 cells expressing GFP, GFP-Etk or a mutant GFP-Etk fusion lacking the PH domain (GFP-EtkΔPH) were stimulated with 100 ng/ml HRGβ for 10 min at 37 °C, fixed, mounted and examined by confocal microscopy. In cells expressing GFP or GFP-Etk, HRGβ stimulation resulted in membrane ruffling, and formation of lamellipodia. While GFP remained predominantly cytosolic in both unstimulated and HRGβ-stimulated MCF-7 cells, HRGβ stimulation of MCF-7 cells expressing GFP-Etk resulted in increased membrane localization of the GFP-Etk fusion protein (Fig. 7). In contrast, GFP-EtkΔPH remained cytosolic in MCF-7 cells, even following HRGβ stimulation. These results suggest that HRGβ stimulation enhances membrane localization of Etk via a mechanism requiring the PH domain of Etk. This is consistent with our overall hypothesis that HRGβ-mediated activation of Etk involves PI 3-K-dependent membrane localization of Etk. We are now extending these studies to determine whether Etk localizes specifically to membrane microdomains following HRGβ stimulation.

- Analyze the role of PI 3-K in the membrane recruitment of Etk upon HRGβ and EGF stimulation by membrane fractionation techniques and confocal microscopy.

We utilized PI 3-K inhibitors to analyze the role of PI 3-K in HRGβ-mediated increases in membrane localization of Etk. Stimulation of MDA-MB-435 cells with HRGβ in the presence of either wortmannin or LY294,002 resulted in a reduction in the amount of Etk found in membrane fractions (Fig. 6), suggesting that HRGβ-mediated increases in Etk membrane localization require PI 3-K. Our confocal microscopy results also demonstrated that HRGβ-mediated membrane localization of Etk is dependent on the PH domain of Etk. Since our phospholipid binding assay indicates that the PH domain of Etk can interact specifically with PI(3,4,5)-$P_3$, the major lipid product of PI 3-K, this suggests that HRGβ-mediated activation of PI 3-K is critical to HRGβ-mediated membrane localization of Etk.

AIM 2. TO DETERMINE THE ROLE OF ETK ON HRG AND EGF MEDIATED INDUCTION OF INTEGRIN ADHESIVENESS AND INTEGRIN DEPENDENT MIGRATION OF MDA-MB-435 BREAST CANCER CELLS.

- Develop eGFP bicistronic vectors expressing wild type and mutant forms of Etk.

  This task was completed in the first year.

- Analyze effects of expression of wild type and mutant Etk constructs on integrin-dependent adhesion and migration of MDA-MB-435 breast cancer cells.

Initial studies on the effects of wild type and mutant Etk on adhesion were performed in the first year and are described in the first year report for this grant. Further studies using additional mutant Etk constructs on cell migration and adhesion will be performed during the third year.

- Analyze effects of expression of wild type and mutant forms of Etk constructs on expression of ß1 integrin activation epitopes.

  This task was not addressed during the second year.

- Identify domains of Etk critical for HRGβ- and EGF- mediated increases in cell adhesion and migration.

  This task was not addressed during the second year.

- Determine if membrane targeting of Etk is sufficient to induce adhesion and migration of breast cancer cells.

Over the past year we have developed the methods and tools for the determination of membrane targeting of Etk following growth factor stimulation. We have successfully shown that HRGβ stimulation of MCF-7 cells transiently expressing GFP –Etk fusion proteins results in membrane localization of WT Etk and not Etk lacking the PH domain. These constructs will be tested for their functional effects on adhesion and migration of breast cancer cells over the next year.

AIM 3. TO DETERMINE THE ROLE OF ETK IN REGULATING GROWTH FACTOR-INDUCED MODIFICATIONS OF THE ACTIN CYTOSKELETON IN BREAST CANCER CELLS.

- Determine the role of Etk in regulating HRGβ- and EGF- induced polymerization of the actin cytoskeleton.

We have previously established the conditions for analyzing growth factor stimulation by flow cytometry and by confocal microscopy. Conditions for transient expression of Etk in breast cancer cells as well as optimizing the conditions for HRGβ stimulation were established as described in Aim 2. These approaches will be utilized to complete this task in the coming year.

- Characterize the interaction between Etk and N-WASP.

This task was not addressed during the second year.

## KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated differential expression of Etk in metastatic versus nonmetastatic breast cancer cell line
- Demonstrated rapid, transient activation of Etk following HRGβ stimulation of MDA-MB-435 breast cancer cells.
- Demonstrated HRGβ-mediated tyrosine phosphorylation of transiently expressed wild-type Etk, but not dominant-negative Etk, in MCF-7 breast cancer cells.
- Demonstrated binding of the PH domain of Etk to the PI 3-K lipid product PI(3,4,5)-$P_3$. Demonstrated that binding of Etk requires the arginine residue at position 29 in the PH domain of Etk. Demonstrated that mutation of glutamic acid at position 42 enhances binding of Etk to PI(3)-P.
- Demonstrated that HRGβ stimulation enhances membrane localization of Etk in MCF-7 cells.
- Demonstrated that HRGβ-mediated membrane localization of Etk in MCF-7 cells requires the PH domain of Etk.

## REPORTABLE OUTCOMES

Abstracts/Presentations

**Mbai, F.N. Qiu, Y. and Shimizu, Y.** Growth factor regulation of the Etk tyrosine kinase in breast cancer cells. Department of Defense Breast Cancer Research Program Meeting: Era of Hope, September 25-28$^{th}$ 2002.

## CONCLUSIONS

In the second year of this three year proposal, we have focused on the effects of HRGβ stimulation on Etk tyrosine phosphorylation and membrane localization, and the role of PI 3-K in HRGβ-mediated effects on Etk. These studies have demonstrated that HRGβ stimulation results in a rapid, transient increase in tyrosine phosphorylation of Etk, as well as increased membrane localization of Etk. In addition, PI 3-K inhibitors block HRGβ-induced increases in Etk tyrosine phosphorylation and membrane localization. Consistent with the role of PI 3-K in the regulation of Etk by HRGβ in breast cancer cells, we demonstrated that the PH domain of Etk mediates binding of Etk to the PI 3-K lipid product PI(3,4,5)-$P_3$. We have also initiated confocal microscopy experiments during the past year, and have shown that the PH domain of Etk is critical for HRGβ-mediated membrane translocation of Etk. Finally, we have shown a relationship between endogenous Etk expression and the metastatic capacity of several well characterized breast cancer cell lines. Overall, these studies have provided further evidence in support of our hypothesis of a function for Etk in the regulation of breast cancer cell adhesion

and migration following HRGβ stimulation. Our proposed plan for the third and final year of support will be to focus our efforts on the functional analysis of the role of Etk in regulating HRGβ-dependent effects on breast cancer cell adhesion and migration. The results obtained during the second year of this work have set the stage for these studies, as we now have the necessary expression constructs and other methodologies to complete these functional studies. In addition to the approved plan of expressing various Etk constructs in MDA-MB-435 cells, which are metastatic and express endogenous Etk, we also propose to determine whether expression of Etk in MCF-7 cells, which are not metastatic and do not express endogenous Etk, will enhance the adhesion and migration of MCF-7 cells in response to HRGβ.

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## APPENDICES

## FIGURES AND FIGURE LEGENDS

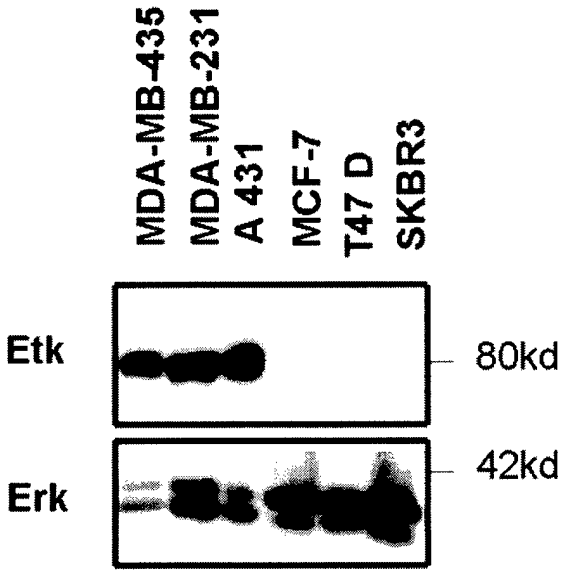


**Figure 1. Etk expression in breast cancer cell lines.** Cells were lysed in RIPA buffer and 50 μg protein separated by SDS-PAGE. Western blotting analysis was performed with an anti-Etk antibody (top panel). The membrane was then stripped and re-probed for an anti-ERK antibody as a loading control (lower panel).

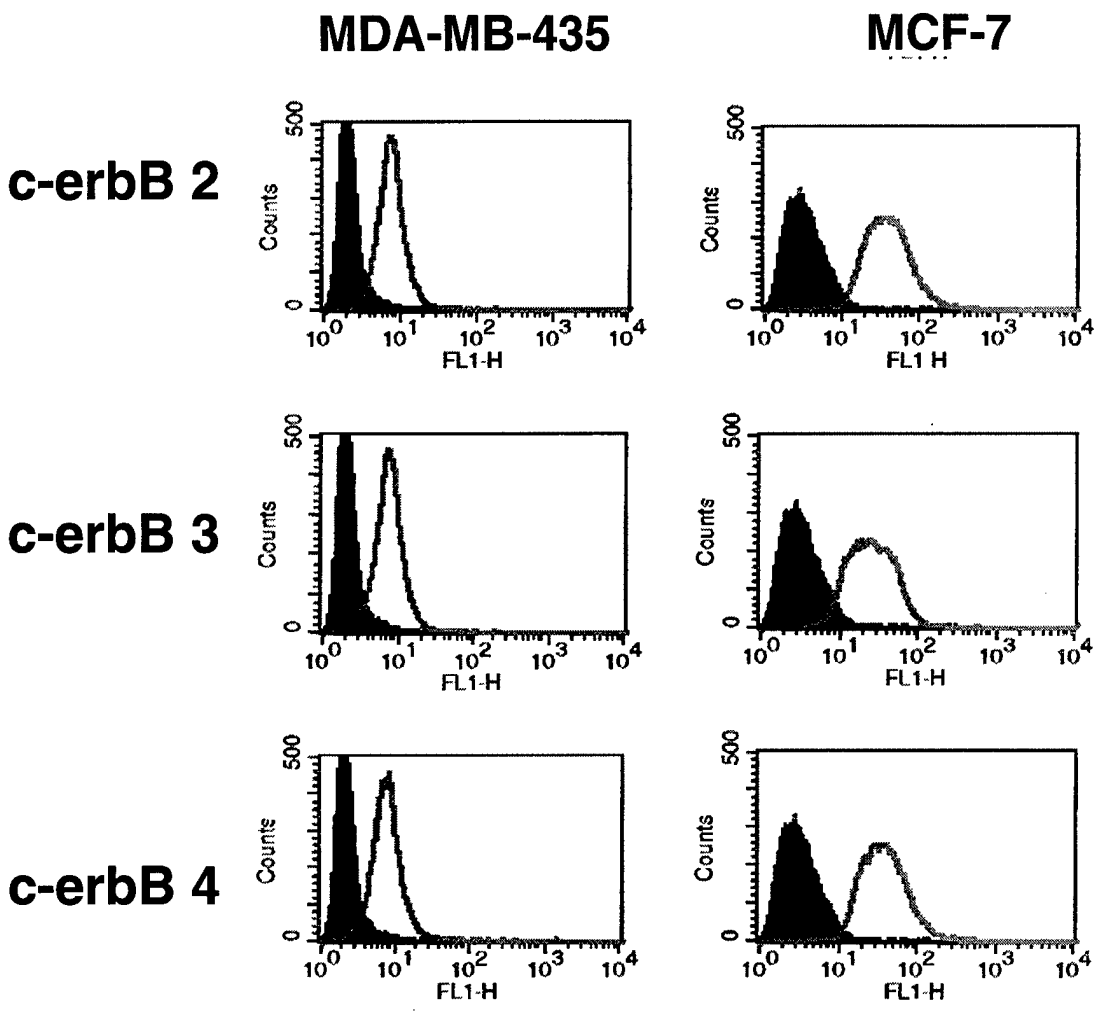


**Figure 2. Expression of c-erbB2, c-erbB3 and c-erbB4 on MDA-MB-435 cells and MCF-7 cells.** Flow cytometric analysis was performed as described in Experimental Methods. Filled histograms indicate negative control staining with no primary antibody.

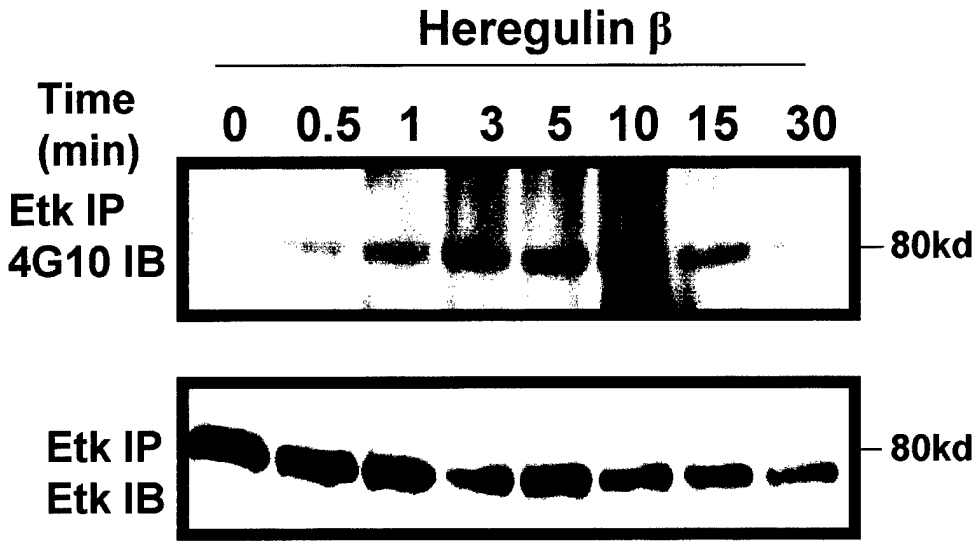


**Figure 3. HRGβ-mediated tyrosine phosphorylation of Etk.** MDA-MB-435 cells were serum starved overnight and stimulated with 100 ng/ml HRGβ in PBS/0.1% BSA. Cells were lysed immediately and Etk was immunoprecipitated using an anti-Etk polyclonal antibody. Equal volumes of the immunoprecipitates were separated on a 7.5% SDS-polyacrylamide gel followed by Western blotting analysis with the anti-phosphotyrosine mAb 4G10 (top panel) or an anti-Etk antibody (bottom panel).

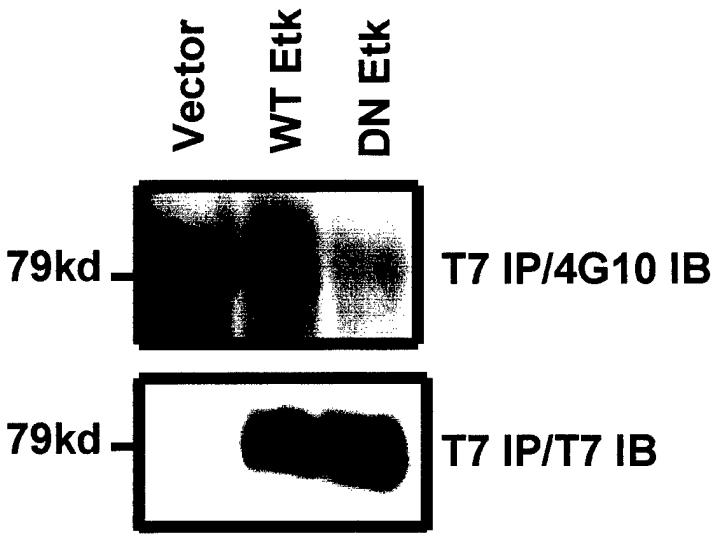


**Figure 4. HRGβ-mediated tyrosine phosphorylation of Etk expressed in MCF-7 cells.** MCF-7 cells were transfected by electroporation with expression vector (pIRES), pIRES T7 tagged Etk and pIRES T7 tagged DN Etk and cultured for 2 days as described in Experimental Methods. Cells were serum starved overnight and then stimulated with 100 ng/ml HRGβ for 10 min., lysed and immunoprecipitated with anti T7 antibody and anti-mouse IgG Sepharose beads. Equal volumes of the immunoprecipitates were separated by SDS-PAGE followed by Western blotting with the anti phosphotyrosine antibody 4G10 (top panel). The membrane was stripped and re-probed with an anti-T7 mAb (lower panel).

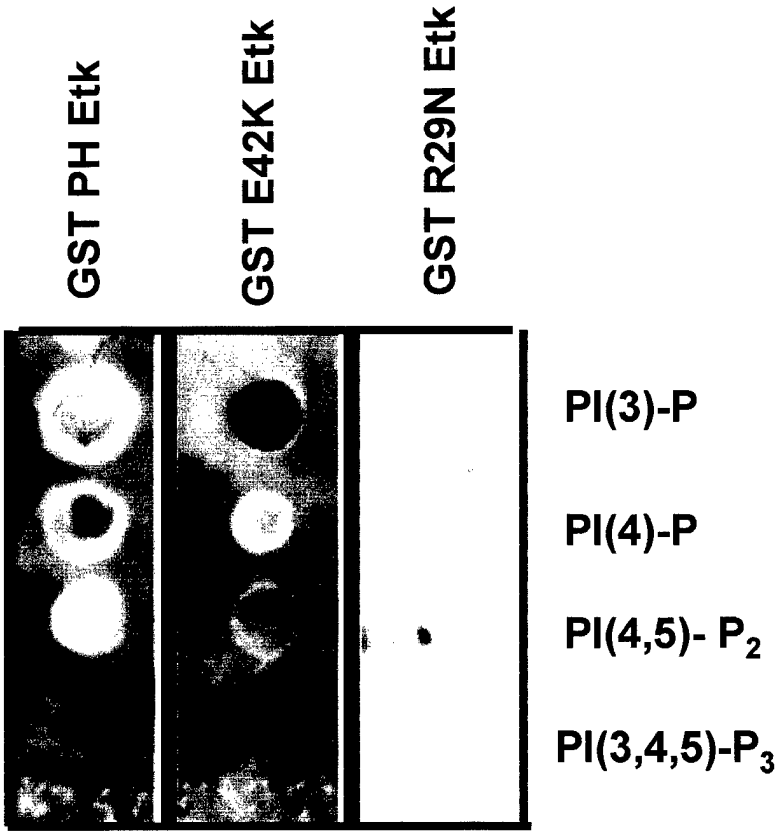


**Figure 5. Phospholipid binding profile of GST-Etk fusion proteins.** GST fusion protein binding to nitrocellulose membranes spotted with 5 μg of purified PI(3)P, PI(4)P, PI(4,5)-$P_2$ or PI(3,4,5)-$P_3$ was assayed as described in Experimental Methods.

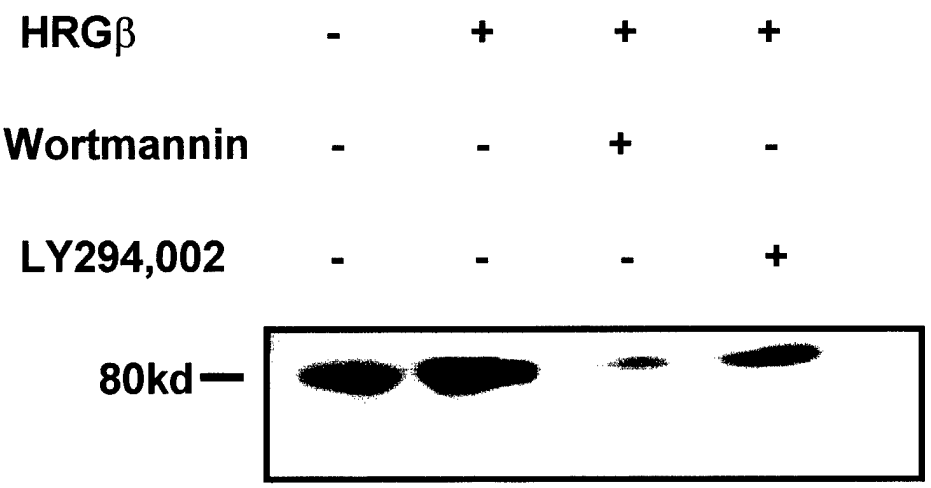


**Figure 6. Effect of PI 3-K inhibitors on HRGβ-mediated membrane translocation of Etk.** MDA-MB-435 cells were stimulated with 100 ng/ml HRGβ or 100 ng/ml HRGβ and wortmannin or LY294,002 for 10 min at 37°C and lysed immediately thereafter. Membrane fractions were obtained as described in Experimental Methods, separated by SDS-PAGE, and analyzed by Western blotting with an anti-Etk antibody.

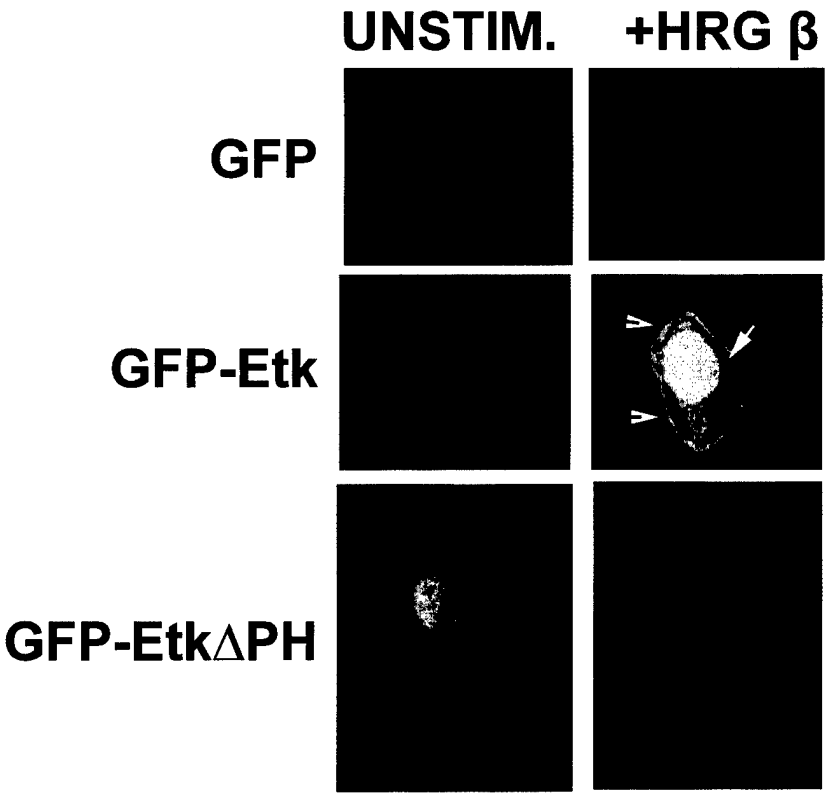


**Figure 7. HRGβ-mediated membrane translocation of Etk in MCF-7 cells.** MCF-7 cells were transfected with the eGFP vector alone, peGFP-Etk or peGFP-EtkΔPH. Cells were stimulated with 100 ng/ml HRGβ for 10 min at 37°C and processed for confocal microscopy as described in Experimental Methods.